

Chromosomal Enrichment and Activation of the Aurora B Pathway Are Coupled to Spatially Regulate Spindle Assembly

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SUMMARY

Chromatin-induced spindle assembly depends on regulation of microtubule-depolymerizing proteins by the chromosomal passenger complex (CPC), consisting of Incenp, Survivin, Dasra (Borealin), and the kinase Aurora B, but the mechanism and significance of the spatial regulation of Aurora B activity remain unclear. Here, we show that the Aurora B pathway is suppressed in the cytoplasm of *Xenopus* egg extract by phosphatases, but that it becomes activated by chromatin via a Ran-independent mechanism. While spindle microtubule assembly normally requires Dasra-dependent chromatin binding of the CPC, this function of Dasra can be bypassed by clustering Aurora B-Incenp by using anti-Incenp antibodies, which stimulate autoactivation among bound complexes. However, such chromatin-independent Aurora B pathway activation promotes centrosomal microtubule assembly and produces aberrant achromosomal spindle-like structures. We propose that chromosomal enrichment of the CPC results in local kinase autoactivation, a mechanism that contributes to the spatial regulation of spindle assembly and possibly to other mitotic processes.

INTRODUCTION

The microtubule-containing bipolar spindle drives eukaryotic chromosome segregation. Spindle microtubules are assembled from both chromosomes and centrosomes, and it is thought that local control of microtubule assembly by chromosomes is important for robust and accurate bipolar spindle formation (reviewed in Bastiaens et al., 2006).

At least three pathways contribute to chromatin-induced spindle assembly, the best characterized involving the small GTPase Ran. At M phase, chromosomes

organize the localized production of Ran-GTP through chromatin-associated RCC1, the Ran guanine nucleotide exchange factor (reviewed in Harel and Forbes, 2004). Peri-chromosomal Ran-GTP then binds to importin β , inducing the release of bound cargoes such as TPX2, NuMA, NuSAP, and Rae1, which promote microtubule assembly (Blower et al., 2005; Gruss et al., 2001; Nachury et al., 2001; Ribbeck et al., 2006; Wiese et al., 2001).

A second pathway involves regulation of the small microtubule-destabilizing protein Op18 (Stathmin), the activity of which has been associated with α/β -tubulin dimer sequestration and direct promotion of microtubule catastrophe (reviewed in Cassimeris, 2002). Phosphorylation of Op18 is induced by chromatin at M phase, and at least three different phosphoacceptor sites are involved in inactivation of its microtubule-destabilizing activity: serine 16 (S16), serine 25, and serine 39 (Andersen et al., 1997). While the latter two are potential Cdk1 target sites, the physiological mitotic kinase for S16 remains unclear. Recently, Gadea and Ruderman (2006) showed that Op18 hyperphosphorylation induced by sperm nuclei depends on Aurora B.

We previously described a third pathway required for chromatin-dependent spindle formation (Sampath et al., 2004) that involves the “chromosomal passenger complex” (CPC). The CPC, which in vertebrates consists of Aurora B, Incenp, Dasra A/B (Borealin), and Survivin (Figure 1A), shows a dynamic localization pattern throughout M phase and plays important roles in promoting proper kinetochore-microtubule attachment, spindle formation, spindle checkpoint signaling, and cytokinesis (Gadea and Ruderman, 2005, 2006; Sampath et al., 2004; Vagnarelli and Earnshaw, 2004). How Aurora B activity can be regulated spatially and otherwise to control such a diverse set of processes nevertheless remains an outstanding question. Such regulation might be achieved through the function of other CPC components; it has been reported that a C-terminal domain of Incenp can allosterically activate Aurora B kinase activity (Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001; Sessa et al., 2005), while the Dasra proteins and Survivin can regulate Aurora B localization to centromeres (Carvalho et al., 2003; Gassmann et al., 2004; Lens et al., 2003; Romano et al., 2003; Sampath et al., 2004; Vader et al., 2006).

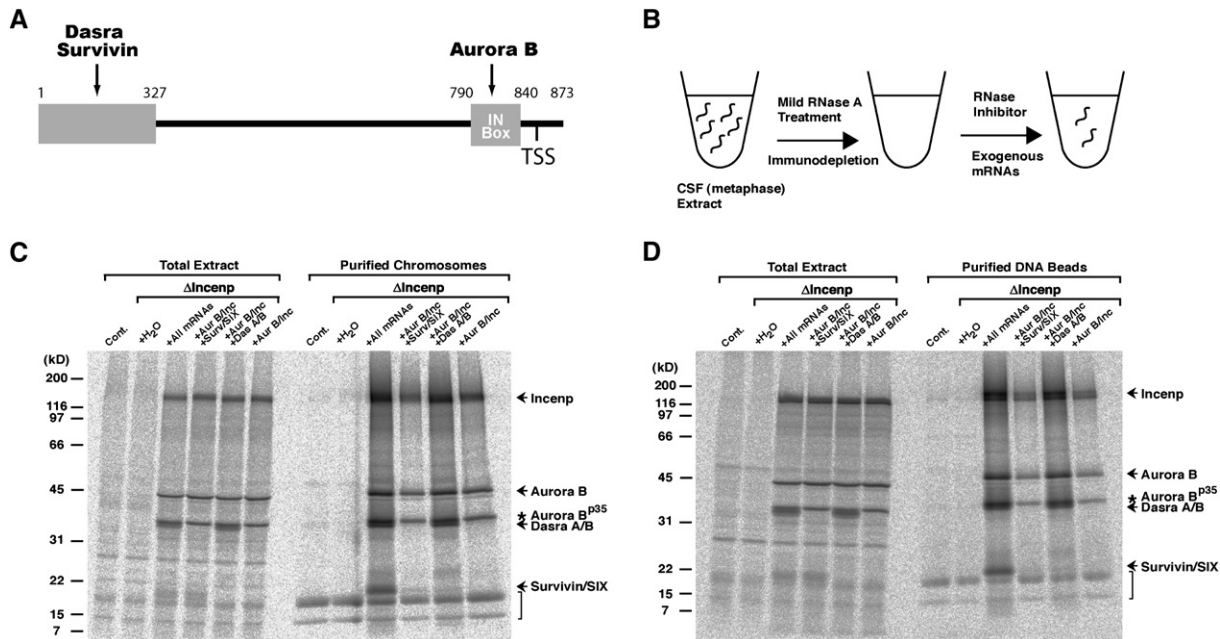


Figure 1. Dasra Proteins Are Required for Efficient Binding of the CPC to Chromatin

(A) Domain layout of *Xenopus* Incenp. Binding sites for Aurora B, Dasra, and Survivin as well as the location of the TSS motif are shown. (B) Schematic for the reconstitution of CPC-immunodepleted (Δ Incenp) egg extract with mRNA pools encoding CPC components. Cytostatic factor (CSF)-arrested metaphase extracts were treated with a low dose of RNase A, leading to destruction of endogenous mRNAs while sparing ribosomes. The CPC was then depleted by using anti-Incenp antibodies coupled to protein A beads. RNase inhibitor and pools of exogenous mRNAs were then added. Proteins encoded by exogenous mRNAs were translated during incubation at 20°C. (C) Dasra proteins are required for efficient CPC binding to metaphase chromosomes. Control, Δ Incenp, or reconstituted Δ Incenp extracts containing sperm nuclei, 35 S-methionine, and biotin-dUTP were cycled through interphase to metaphase, and biotinylated chromosomes were purified. The combination of mRNAs is indicated at the top of the lane; "All mRNAs" represent in vitro-transcribed mRNAs of Aurora B, Incenp, Dasra A, Dasra B, Survivin, and SIX. Copurification of labeled proteins was analyzed by SDS-PAGE and was quantified by using a PhosphorImager. The asterisk indicates the Aurora B^{p35} species, a product of internal translation initiation produced from exogenous Aurora B mRNA (T.A.M. and S.C.S., unpublished data). The arrow indicates Dasra proteins. Note that copurified labeled histones (indicated by the brackets) are evident due to incomplete degradation of their abundant mRNAs during RNase treatment and indicate equal chromosome recovery. (D) Dasra proteins are required for efficient CPC binding to chromatin beads. Control, Δ Incenp, or reconstituted Δ Incenp extracts containing DNA beads and 35 S-methionine were cycled through interphase to metaphase, and the beads were isolated and analyzed as described above.

Downstream of Aurora B is the microtubule depolymerase MCAK, a kinesin 13 family member that catalytically depolymerizes microtubule ends (Desai et al., 1999b; Hunter et al., 2003). Phosphorylation of MCAK inhibits its microtubule-depolymerizing activity, and nonphosphorylatable MCAK mutants are accordingly unable to support bipolar spindle assembly (Andrews et al., 2004; Lan et al., 2004; Ohi et al., 2004).

Here, we demonstrate a critical role for chromatin-mediated activation of the Aurora B pathway during spindle assembly. We demonstrate that the Aurora B pathway is normally suppressed in the cytosol of *Xenopus* egg extract, but that it becomes activated by chromatin via a Ran-independent mechanism. Using an mRNA-dependent system for CPC reconstitution, we further show that Dasra A is required for loading of the CPC onto chromatin, and therefore chromatin-dependent activation of the Aurora B pathway. Surprisingly, we find that antibodies recognizing the C terminus of Incenp cluster the CPC and can effectively bypass the requirement for Dasra proteins in spindle microtubule assembly. This activation of the

CPC leads to the formation of abnormal spindle-like structures, including "achromosomal" spindle formation by centrosomes. Our data demonstrate that clustering of the CPC by chromatin activates the Aurora B pathway, which plays a key role in confining spindle assembly to the vicinity of chromosomes.

RESULTS

A System for Reconstitution of the CPC in *Xenopus* Egg Extracts

We previously described the identification of Dasra A and Dasra B, two new components of the vertebrate chromosomal passenger complex (CPC), and we reported that immunodepletion of the CPC from *Xenopus* egg extract with anti-Incenp antibodies leads to failure of bipolar spindle formation around M phase chromatin (Sampath et al., 2004). To test the function of individual CPC components, we adapted the previously described "mRNA-dependent" extract system (Murray and Kirschner, 1989) (Figure 1B). For CPC reconstitution, pools of

in vitro-transcribed CPC mRNAs were added to RNase A-treated, CPC-depleted (Δ Incenp) extracts. Labeling of the reconstituted extracts with ^{35}S -methionine demonstrated efficient translation of all exogenous CPC mRNAs (Figures 1C and 1D, Total Extract), including Aurora B, Incenp, Dasra A, Dasra B, Survivin, and SIX, a second Survivin-related protein in *Xenopus* (Figure S1; see the Supplemental Data available with this article online) (Song et al., 2003). Immunoblots with antibodies recognizing Incenp, Aurora B, Dasra A, and Survivin indicated that all of these proteins were reconstituted to approximately endogenous levels (data not shown, but see Figures 2C and 5B).

Dasra Proteins Facilitate Loading of the CPC onto Chromatin

To investigate how Dasra proteins contribute to CPC function, we analyzed the ability of the CPC to copurify with chromosomes in the presence or absence of the Dasra proteins. Metaphase chromosomes were purified from reconstituted Δ Incenp extract that had been metabolically labeled with ^{35}S -methionine, and the extent of copurification of the labeled CPC members was examined (Figure 1C). We observed that ~ 9 -fold less Incenp and Aurora B and ~ 25 -fold less Survivin copurified with chromosomes when Dasra proteins were absent than when present. Essentially the same effect was seen on purified chromatin beads lacking centromeric sequences (Figure 1D), indicating that centromeres are not required for Dasra-dependent loading of the CPC onto chromosomes. Consistent with this conclusion, immunofluorescence analyses revealed reduction of Incenp staining on both centromeres and chromosome arms in the absence of Dasra proteins (Figure S2B).

Dasra A, Together with Aurora B and Incenp, Is Required for Spindle Assembly in *Xenopus* Egg Extracts

We previously proposed that the CPC on chromosomes locally controls phosphorylation of MCAK to promote chromatin-induced microtubule assembly (Sampath et al., 2004), suggesting that Dasra proteins, which facilitate CPC binding to chromatin, would be important for spindle assembly. Indeed, we found that CPC depletion led to the expected absence of chromatin-associated microtubules, whereas addition of a pool containing Aurora B, Incenp, and Dasra A was sufficient for rescuing bipolar spindle assembly (Figure 2). Furthermore, Aurora B, Incenp (data not shown), and Dasra A (Figure 2) are all necessary for the rescue.

A small population ($\sim 11\%$) of DNA-based structures seen in the Aurora B and Incenp condition had "mini-spindles" (Figures S2 and S3) with average lengths of $13 \pm 4 \mu\text{m}$ (versus $38 \pm 6 \mu\text{m}$ for control spindle length). We believe that these mini-spindles reflect the recruitment of a small amount of residual undepleted Dasra proteins onto the chromosomes (Figure S3). Indeed, when a deletion mutant of Incenp (Incenp $^{\Delta 1-327}$), which retains binding to Aurora B, but which cannot bind to Dasra proteins or Survivin/SIX (Figure 1A and Figure S1) (Bolton et al.,

2002), was used, an even smaller population of spindles exists in the Aurora B and Incenp $^{\Delta 1-327}$ condition (3%; Figure 2B) in the presence or absence of Dasra A. This result reflects the importance of the Incenp-Dasra A interaction for CPC binding to chromosomes and spindle assembly (Figures S3 and S4).

Hyperphosphorylation of Op18 Is Dependent on the CPC

The results described so far suggest the close link between chromosomal localization of the CPC and spindle assembly. We therefore asked if chromosomes activate the Aurora B pathway, and we sought useful markers that indicate the level of pathway activation. We considered that Op18 hyperphosphorylation might be a good candidate, because it is induced by chromatin (Andersen et al., 1997), and because Ser16, a critical site of Op18 phosphorylation, resides within an Aurora target consensus site (Ferrari et al., 2005).

To determine whether Aurora B can phosphorylate Op18, the kinase activity of immunoprecipitated CPC was examined toward recombinant Op18 and Op18-S16A mutant proteins. Plx1 and control IgG immunoprecipitations were used as controls for specificity, and myelin basic protein (MBP) was used as a positive control for kinase activity. Immunoprecipitated CPC robustly phosphorylated both MBP and wild-type Op18, whereas phosphorylation of Op18-S16A was ~ 14 -fold less than that of wild-type Op18, demonstrating that S16 is the major site of Aurora B-dependent phosphorylation in vitro (Figure 3A). In contrast, Plx1 had only relatively weak activity against both wild-type and S16A Op18 (Figures 3A).

Previous studies have shown that the addition of sperm nuclei, purified centrosomes, or DNA beads to metaphase egg extracts induces hyperphosphorylation of Op18 (Andersen et al., 1997; Kuntziger et al., 2001). We similarly observed that the most hyperphosphorylated (slowest migrating) form of Op18 accumulated in a dose-dependent manner under all three conditions, but the formation of this hyperphosphorylated species was completely blocked in Δ Incenp extract (Figure 3B). Although it has been suggested that Plx1 mediates Op18 phosphorylation in *Xenopus* egg extract (Budde et al., 2001), depletion of Plx1 showed a relatively minor effect (Figure S5). Altogether, these data demonstrate that Aurora B leads to chromatin- and centrosome-induced hyperphosphorylation of Op18.

Op18 and Histone H3 Phosphorylation Are Reporters of Aurora B Pathway Activation by Chromatin, Centrosomes, and Stabilized Microtubules

As Op18 hyperphosphorylation stimulated by taxol-induced microtubule stabilization (Kuntziger et al., 2001) also requires the CPC (Figure S6), we investigated whether hyperphosphorylation induced by chromatin and centrosomes is caused by microtubules assembled by these structures. We observed no effect of nocodazole on Op18 phosphorylation (Figure 3C, left), even though microtubule polymerization was clearly inhibited (Figure 3C,

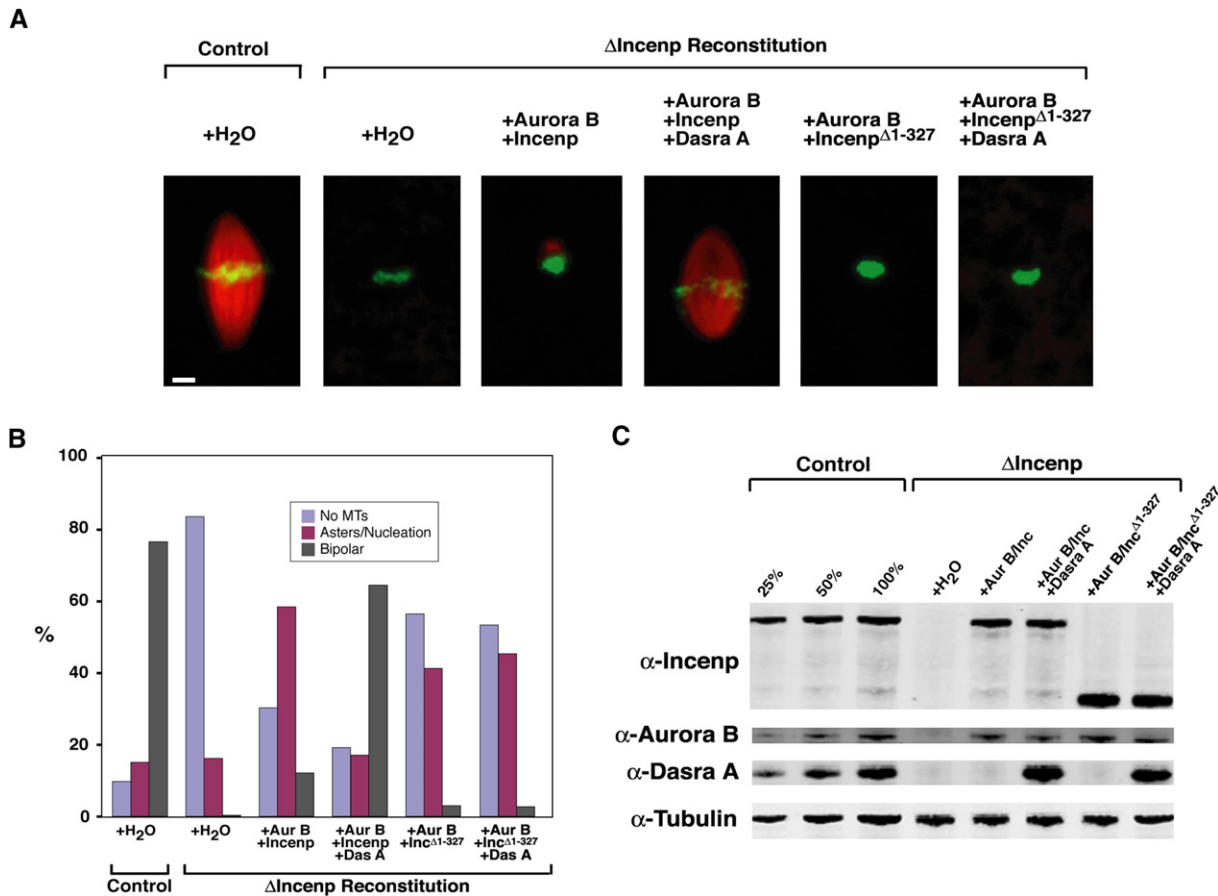


Figure 2. Dasra A Is Required for Spindle Assembly

(A) Spindle assembly in reconstituted egg extracts. Control or Δ Incnp extracts were reconstituted as indicated and were cycled through interphase to metaphase with sperm nuclei, and rhodamine-tubulin (red) was added to visualize microtubules; extracts were fixed 60 min after entry into metaphase. Hoechst 33258 (green) was used to visualize DNA. The scale bar is 10 μ m.

(B) Quantitation of structures formed in the extracts described in (A). At least 200 chromosome-containing samples were analyzed per sample.

(C) Western blot of samples from (A).

right). In addition, phosphorylation of histone H3 serine 10 (H3S10), a canonical chromosomal substrate of Aurora B (Murnion et al., 2001), closely mirrored the pattern of Op18 hyperphosphorylation (Figure 3C and Figure S6). Thus, Op18 and H3S10 phosphorylation can be thought of as markers of Aurora B activity, which is suppressed in the cytoplasm of *Xenopus* egg extracts, but can be induced by chromatin in a microtubule-independent manner.

Anti-Incnp Antibodies Activate the Aurora B Pathway by Increasing the Local Concentration of the CPC

We next sought to identify a simple manipulation that activates the CPC in the cytoplasm independent of chromatin, centrosomes, or microtubules. We considered that cytoplasmic phosphatase activity may dampen the kinase activity of Aurora B, but the high local concentration of the CPC on chromatin facilitates autostimulation of the CPC. Indeed, incubation of beads coated with anti-Incnp, anti-Dasra A, or anti-Aurora B with metaphase extract,

which should increase the local concentration of the CPC, induced Op18 hyperphosphorylation (Figure 4A). This effect was specific to antibodies against components of the CPC, as anti-Plx1 or anti-xKid antibodies did not induce Op18 hyperphosphorylation.

The simplest explanation of the result described above is that antibody-mediated clustering facilitates phosphorylation among the CPCs, which stimulates the kinase activity. It has been shown that phosphorylation of the C-terminal TSS motif in Incnp is carried out by Aurora B and is important for full activation for the kinase activity (Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001; Sessa et al., 2005; Yasui et al., 2004). To test if antibody-mediated clustering induces phosphorylation of these sites, anti-Incnp-coated beads were incubated with metaphase extracts at 4°C or 20°C, and then the CPC was purified with beads. At 4°C, a condition predicted to diminish enzymatic activity, the CPC can still associate with antibody beads, but a smaller degree of Op18 hyperphosphorylation was observed when compared to

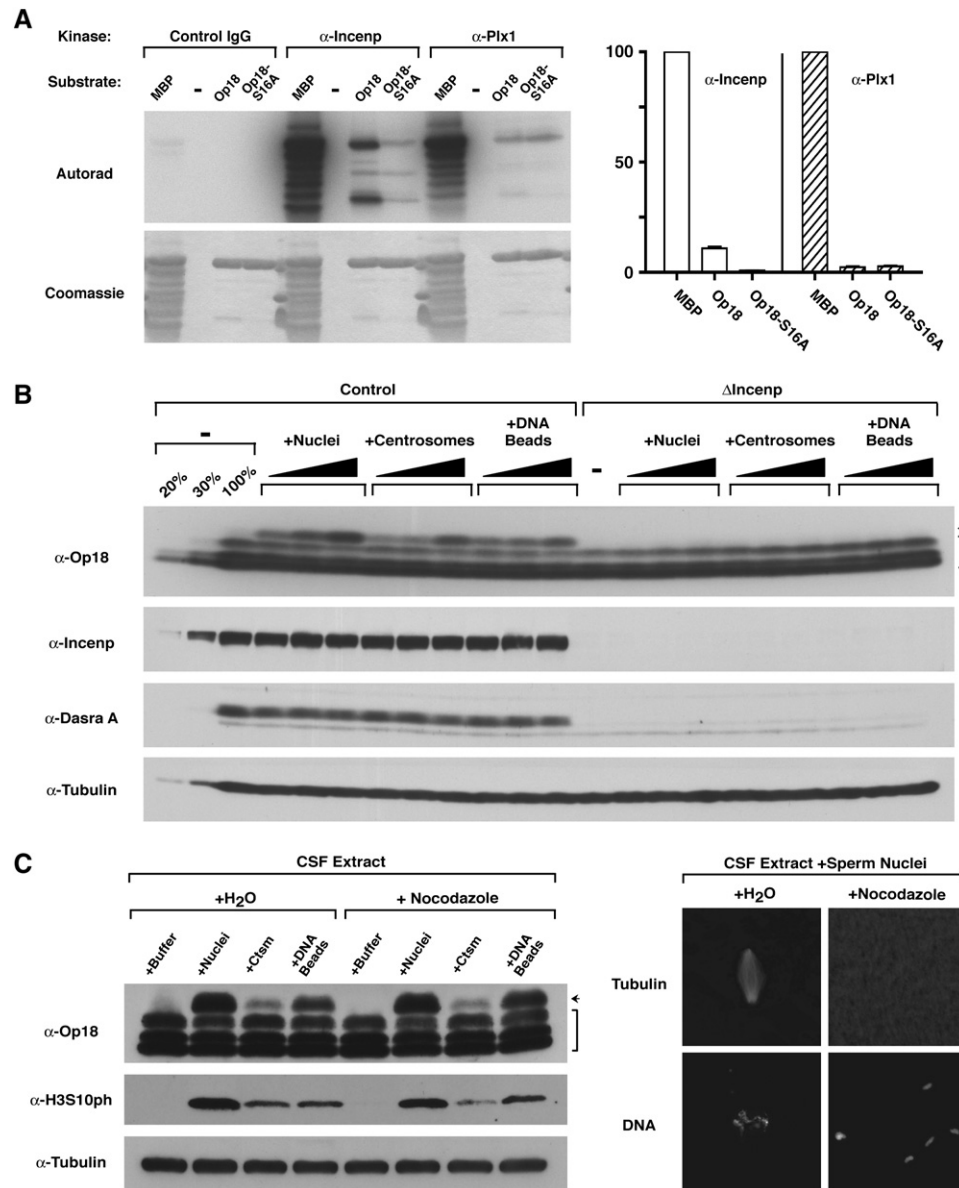


Figure 3. Chromatin- and Centrosome-Mediated Op18 Hyperphosphorylation Depend on the CPC

(A) In vitro kinase assay against recombinant Op18. The CPC and Plx1 were immunoprecipitated from CSF extract with anti-Incenp and anti-Plx1 antibodies, respectively, and they were tested for kinase activity with recombinant 6xHis-Op18 or 6xHis-Op18-S16A as a substrate. Control IgG precipitation was used as a negative control, and myelin basic protein (MBP) was used as a positive control for the kinase assays. Quantitation (right) was performed after SDS-PAGE and exposure to a PhosphorImager; all values are normalized against activity toward MBP. The mean and range of two experiments are shown.

(B) CPC-dependent Op18 phosphorylation is induced by chromatin and centrosomes. Increasing amounts of sperm nuclei (500, 2,000, or 10,000/ μ l), purified centrosomes (250, 1,000, or 10,000/ μ l) or DNA beads (1,000, 2,000, 5,000 beads/ μ l extract, respectively) were added to control or Δ Incenp extract and were cycled through interphase to metaphase. Samples were taken 45 min after entry into metaphase and were analyzed by western blot. The arrow indicates the slowest-migrating, hyperphosphorylated form of Op18, and the brackets indicate faster-migrating Op18 species with various states of phosphorylation.

(C) Centrosomes and chromatin induce CPC-dependent, but microtubule-independent, phosphorylation of both Op18 and H3S10. Sperm nuclei (Nuclei; 10,000/ μ l), centrosomes (Ctsm; 3,000/ μ l) or DNA beads (5,000/ μ l) were added to CSF extracts containing rhodamine-tubulin in the presence or absence of 33 μ M nocodazole. Samples were taken after 100 min and were analyzed by western blot. Representative images of structures assembled in the presence or absence of nocodazole are shown at the right for tubulin (top) and Hoechst 33258 (bottom).

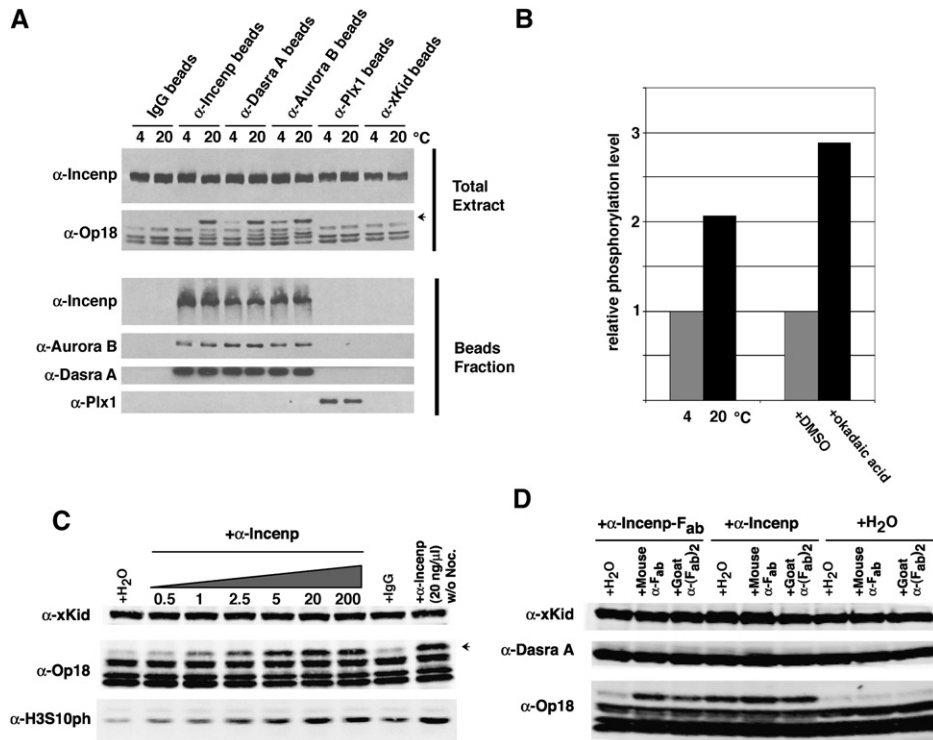


Figure 4. Incubation of Anti-Incenp Antibodies with Egg Extracts Stimulates Op18 Phosphorylation

(A) Indicated antibodies (2 μg) were crosslinked to protein A beads and incubated with 20 μl CSF extract containing nocodazole at 4°C or 20°C for 85 min. Total input (extracts and beads) and the isolated beads fraction were analyzed by western blot. Antibodies against CPC components induced Op18 hyperphosphorylation (indicated by an arrow) when incubated at 20°C.

(B) Relative phosphorylation of Incenp tryptic peptide (847–862) TSSAVWHSPPLSSNR isolated from extracts incubated at 4°C and 20°C with anti-Incenp antibodies (left) analyzed by mass spectrometry. The relative phosphorylation from extracts treated with 0.5 μM okadaic acid or DMSO control is also shown (right).

(C) The indicated amount (ng/μl) of anti-Incenp antibodies or 200 ng/μl control IgG was incubated with 30 μl CSF extract at 20°C for 60 min in the presence of nocodazole, except for the last lane. The Op18 and H3S10 phosphorylation levels were measured by quantitative western blot analysis. An arrow indicates the hyperphosphorylated form of Op18. xKid was used as a loading control.

(D) F_{ab} fragment of anti-Incenp antibody (20.8 ng/μl ≈ 400 nM), native anti-Incenp antibody (70 ng/μl ≈ 400 nM), or water was incubated with CSF extracts together with water, mouse anti-light-chain antibody (30 ng/μl ≈ 200 nM), or goat-anti-(F_{ab})₂ antibody (30 ng/μl ≈ 200 nM) at 20°C for 60 min in the presence of nocodazole. Op18 hyperphosphorylation was analyzed by quantitative western blot.

samples incubated at 20°C (Figure 4A). Therefore, we speculated that complex isolated from extracts preincubated with anti-Incenp beads at 4°C and 20°C represent the pre- and postactivated status of the CPC, respectively. The phosphorylation of the Incenp TSS motif isolated from beads preincubated at 20°C was increased by 2-fold when compared to the 4°C sample, as analyzed by quantitative mass spectrometry (Figure 4B and Figure S7). Moreover, addition of 0.5 μM okadaic acid to metaphase extracts, previously shown to inhibit PP2A activity by 50% (Félix et al., 1990), increased Incenp TSS phosphorylation by 2.9-fold. Recently, cytoplasmic suppression of TSS phosphorylation was also shown by using an anti-phospho-S850 Incenp antibody (Knowlton et al., 2006). These data suggest that the TSS motif is underphosphorylated in the cytoplasm due to phosphatase activity, but that clustering of the CPC increases the strength and lifetime of this signal, in turn increasing the net Aurora B kinase activity in the system.

To our surprise, addition of soluble anti-Incenp antibody to control extract induced Op18 hyperphosphorylation and H3S10 phosphorylation in a dose-dependent manner in both the presence and absence of nocodazole (Figures 4C and 7A). To examine if this anti-Incenp antibody induces Aurora B pathway activation by increasing the local concentration of the CPC, we tested if the F_{ab} fragment of the anti-Incenp antibody (which contains one binding site per molecule instead of two) could increase Op18 hyperphosphorylation (Figure 4D). The F_{ab} fragment was not able to induce hyperphosphorylation of Op18, but this activity was restored when the F_{ab} fragment was supplemented with secondary antibodies recognizing the F_{ab} fragment (mouse anti-light chain and goat anti-(F_{ab})₂), demonstrating that the F_{ab} fragment can still recognize Incenp, but such binding was not sufficient for activation.

We conclude that Aurora B-dependent phosphorylation is stimulated by the anti-Incenp antibody by the forced proximity of Aurora B-containing complexes caused by

the existence of two antigen-binding sites per antibody molecule, and/or by further clustering of antibodies mediated by the F_c fragment. Addition of this antibody can therefore circumvent the need for physiological Aurora B pathway activators in *Xenopus* egg extracts.

Effects of Anti-Incenp Antibodies in Spindle Assembly

Having established that the anti-Incenp antibody can autonomously activate the Aurora B pathway in the cytoplasm, we then investigated if the Aurora B pathway requires local activation by chromosomes to spatially restrict microtubule assembly.

Spindle morphologies were monitored in control extracts; in Δ Incenp extracts supplemented with Aurora B, Incenp, and Dasra A; or in Δ Incenp extracts with Aurora B and Incenp $^{\Delta 1-327}$, in the presence or absence of 2.5, 5, or 10 ng/ μ l anti-Incenp antibody at various time points after entry into M phase (Figure 5 and Figure S8). Incenp $^{\Delta 1-327}$ was employed in place of full-length Incenp to avoid any interaction with undepleted Dasra A. In control extract and Δ Incenp extract complemented with Aurora B, Incenp, and Dasra A, visible microtubule structures were predominantly associated with chromosomes throughout the time course, and the bipolar spindle was the major form at 60 min after entry into M phase (Figure S8). At this point, hyperphosphorylation of Op18 was also observed in these extracts (Figure 5B). In Δ Incenp extracts with no complementation or in extracts complemented with both Aurora B and Incenp $^{\Delta 1-327}$, more than 50% of chromosomes did not associate with any visible microtubules as previously shown (Figures 2 and 5A; Figure S8). Strikingly, in Δ Incenp extract containing Aurora B, Incenp $^{\Delta 1-327}$, and anti-Incenp antibody (2.5 ng/ μ l), the majority (>90%) of chromosomes were associated with microtubules, and about 50% of chromosome-containing structures were bipolar spindles at 45 min (Figure 5A and Figure S8). Furthermore, robust Op18 hyperphosphorylation was observed even in the absence of Dasra A. At later time points (60 and 75 min), in extracts containing anti-Incenp antibody, the bipolarity of spindles was greatly diminished, and spindles were often clustered together. These abnormal structures correlate with the hyperactivation (Figure 5B) and mislocalization of the CPC (Figure S9; the majority of Incenp is localized to microtubules instead of chromosomes), which may affect the spatial regulation of microtubule assembly. This unusual Incenp-microtubule association does not seem to be mediated by anti-Incenp antibodies, since Incenp is preferentially localized to microtubules when its chromosome binding is abrogated upon depletion of Dasra A (Figure S2B).

Another apparent phenotype caused by anti-Incenp antibodies was the presence of achromosomal microtubule structures, including bipolar, spindle-like structures. At 15 min, 13%, 56%, and 56% of achromosomal microtubule structures are bipolar in the presence of 2.5, 5, and 10 ng/ μ l anti-Incenp, respectively. After 45 min, more than 70% of achromosomal structures are bipolar in all cases with anti-Incenp. Anti-Incenp antibodies were colocalized to these achromosomal spindle microtubules, indicating

that the CPC may promote microtubule assembly in part through interaction with them (Figure S9). We assumed that these achromosomal structures are formed by the microtubules nucleated from centrosomes derived from sperm nuclei, as they were not formed when DNA beads were used for spindle assembly instead of sperm nuclei (data not shown). These results indicate that anti-Incenp antibody is able to activate the Aurora B pathway independent of Dasra A and to assemble microtubules that are nucleated from either chromosomes or centrosomes.

Microtubule Assembly by Aurora B Pathway Activation

To avoid the pleiotropic phenotype seen on sperm chromosomes, we quantified the effect of the anti-Incenp antibody by using purified centrosomes as the sole source of microtubule nucleation (Figure 6). Microtubules were highly unstable in control M phase extracts or Δ Incenp extracts complemented with water or Aurora B and Incenp, and the average diameter size of centrosomal microtubule structures was about 10 μ m in all of these extracts (Figures 6A and 6B). In control extract containing the anti-Incenp antibody or in Δ Incenp extract complemented with Aurora B and Incenp together with the anti-Incenp antibody, however, the average diameter of centrosomal microtubule structures was 70–90 μ m, and centrosomal interactions were frequently observed (Figure 6C).

To rule out the possibility that the antibody exerted its effects on microtubules independently of Aurora B by clustering the microtubule-binding domain of Incenp (Wheatley et al., 2001), the anti-Incenp antibody was added to Δ Incenp extract complemented with only Incenp. No centrosomal interactions were seen, and there was no increase in the appearance of astral microtubules around centrosomes (Figures 6A and 6B). Together, these data demonstrate that promotion of microtubule assembly and centrosomal interaction are caused by Aurora B kinase activated by anti-Incenp antibodies.

The Aurora B Pathway Is Activated by a Ran-Independent Mechanism

One prevailing model implies that Ran-GTP locally generated by chromatin is critical for chromatin-induced spindle formation (Caudron et al., 2005; Kalab et al., 2006). To determine if chromatin activates the Aurora B pathway through the Ran-GTP pathway, the Op18 hyperphosphorylation response induced by a broad range of sperm chromosome concentrations was monitored in the presence or absence of 15 μ M RanT24N, which prevents the production of Ran-GTP. As previously shown (Carazo-Salas et al., 1999), RanT24N effectively blocked microtubule assembly from DNA beads (data not shown). However, RanT24N did not reduce the capacity of sperm chromosomes to induce hyperphosphorylation of Op18 (Figure 7A, left panel). Similarly, RanT24N did not show any effect on Op18 phosphorylation induced by anti-Incenp antibody (Figure 7A, right panel). These data demonstrate that the Aurora B pathway can be activated by chromosomes via a Ran-GTP-independent mechanism.

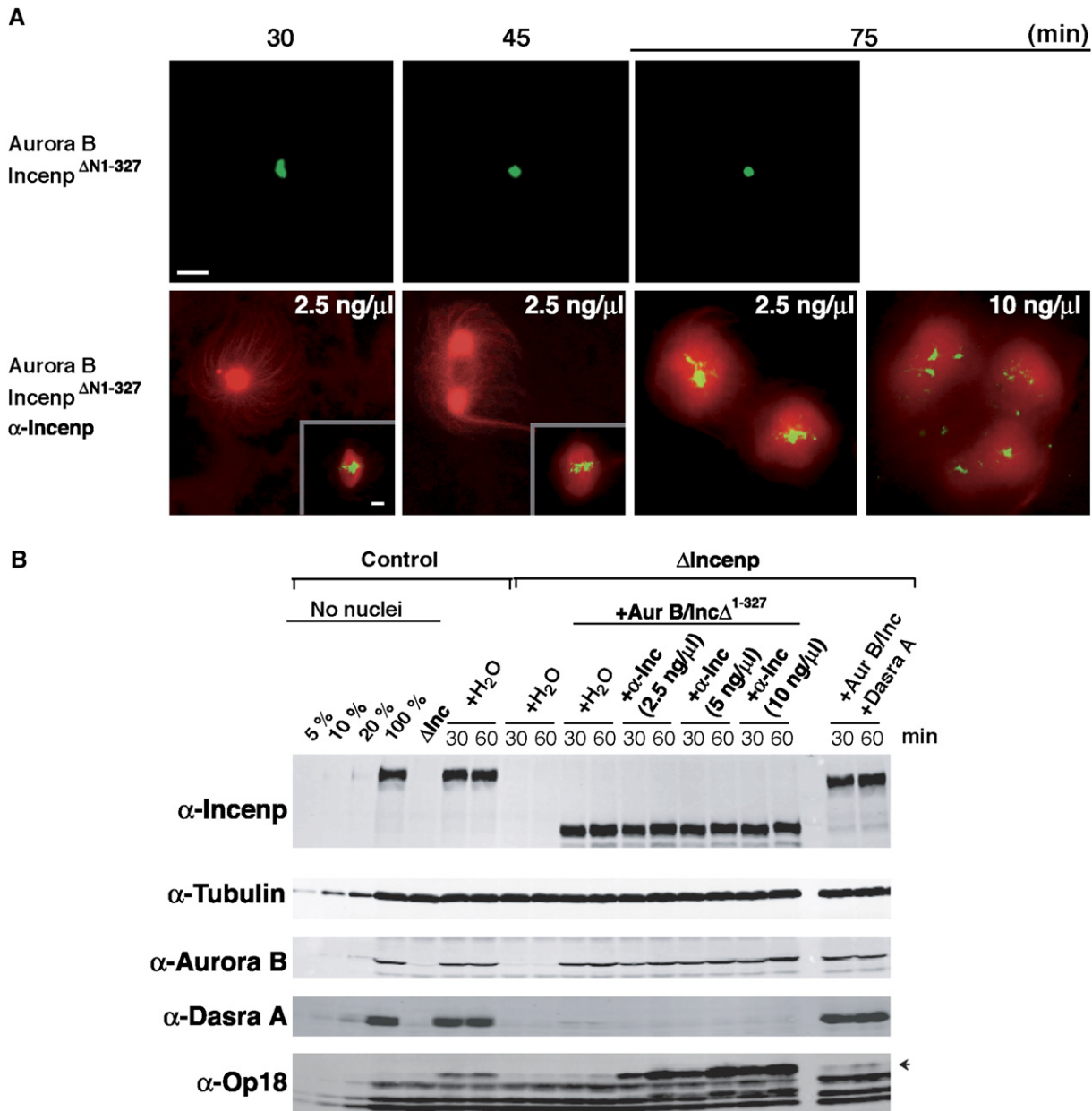


Figure 5. Anti-Incnp Antibodies Bypass the Requirement of Dasra A in Spindle Microtubule Assembly and Induce Aberrant Achromosomal Microtubule Assembly

(A and B) Spindle formation around sperm chromosomes was monitored in control extract or in Δ Incnp extract complemented with either (1) Aurora B/Incnp Δ 1-327 in the presence or absence of anti-Incnp antibody (2.5, 5, or 10 ng/ μ l) or (2) Aurora B/Incnp/Dasra A. Extracts were prepared as described (Figure 2), except that 24 μ g/ml cyclin B Δ 90 was added. A full analysis of each condition is described in Figure S8. (A) Representative images of structures formed at 30, 45, and 75 min after entry into M phase for Δ Incnp extracts containing Aurora B/Incnp Δ 1-327, Aurora B/Incnp Δ 1-327 + 2.5 ng/ μ l anti-Incnp, or Aurora B/Incnp Δ 1-327 + 10 ng/ μ l anti-Incnp. Representative bipolar spindle structures that formed (inset) are shown for the 30 and 45 min time points. Rhodamine-tubulin (red) and Hoechst 33258 (green) were used to visualize microtubules and DNA, respectively. The scale bars are 20 μ m. (B) All samples were analyzed by quantitative western blot at 30 and 60 min after entry into M phase. The left-most lanes represent indicated amounts of control extracts.

Finally, the effect of RanT24N on microtubule assembly induced by anti-Incnp antibodies was examined by monitoring microtubules nucleated from centrosomes, since RanT24N inhibits microtubule nucleation from chromatin. The positive effect of the anti-Incnp antibody on microtu-

bule assembly was not affected by RanT24N (Figure 7B). This result strongly suggests that once microtubules are nucleated, activation of the anti-Incnp-mediated Aurora B pathway can promote their assembly even in the absence of an active Ran pathway.

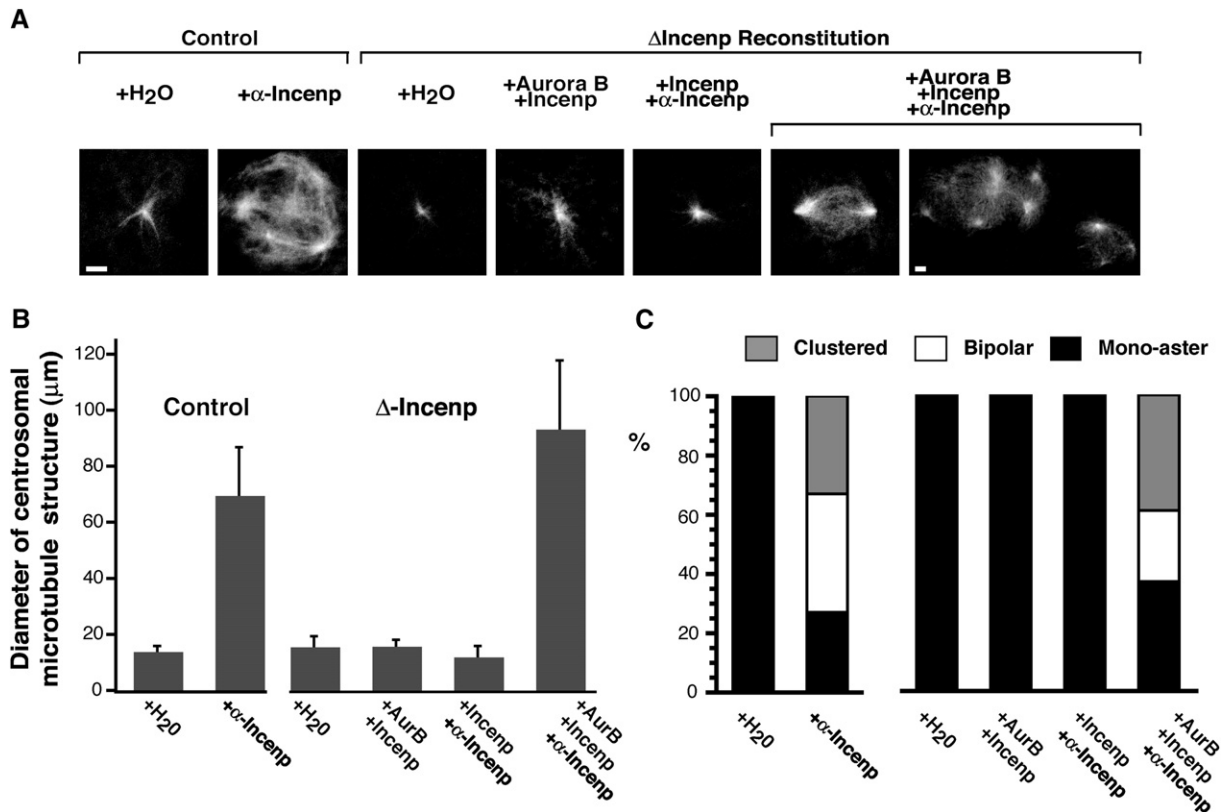


Figure 6. Antibody-Induced Aurora B Pathway Activation Leads to Achromosomal Spindle Formation by Centrosomes

(A–C) Representative fields of microtubules formed in cycled control or Δ Incenp extract containing human centrosomes (400/ μ l) and reconstituted with either water or the indicated CPC components. Where indicated, anti-Incenp antibody (5 ng/ μ l) was added to extracts. (A) All images are shown at equal magnification, except for the last image. Rhodamine-tubulin was added to visualize microtubules. The scale bars are 10 μ m. (B) The average diameter with standard deviation of microtubule structures associated with monoasters in each sample is shown. (C) Aster structures were categorized into “monoaster,” “bipolar,” and “clustered,” and the frequency of each category is presented. At least 110 microtubule-containing structures were counted for each sample.

DISCUSSION

We previously reported that the CPC is required for chromatin-induced spindle assembly, and we proposed that this regulation occurs in part through phosphorylation of the microtubule depolymerase MCAK by Aurora B (Sam-path et al., 2004). Here, we show that Aurora B can regulate another microtubule-depolymerizing protein, Op18. During the course of revising this manuscript, similar results were reported (Gadea and Ruderman, 2006). Using Op18 hyperphosphorylation as an indicator for the Aurora B pathway activation, we demonstrate that the pathway is suppressed in cytoplasm, but is activated by chromatin through a Ran-independent mechanism. Below, we discuss the mechanism and significance of chromatin-induced activation of the Aurora B pathway.

Mechanisms of Aurora B Activation by Chromatin

How does chromatin activate Aurora B-dependent phosphorylation? Four lines of evidence support a model in which Aurora B is activated by increasing the local concentration of CPC molecules on chromatin: (1) Chro-

matin can bind to multiple molecules of the CPC and induce Aurora B pathway activation (Figures 1 and 3); (2) Antibody alone can activate Aurora B kinase activity, and this activity is dependent on having multiple binding sites (Figures 4C and 4D); (3) The responses of Op18 hyperphosphorylation induced by sperm nuclei and antibodies are similar and Ran independent (Figure 7A); (4) Op18 hyperphosphorylation induced by antibody clustering is insensitive to the geometry of attachment (Figure 4A).

Full activation of Aurora B requires Aurora B-mediated phosphorylation of the C-terminal TSS motif of Incenp, and structural analysis suggests that this phosphorylation must occur in trans (Sessa et al., 2005). Thus, the simplest model is that the Incenp TSS motif is actively dephosphorylated in the cytoplasm, but chromatin increases the local concentration of the CPC, resulting in initiation of a positive feedback loop among bound CPC holocomplexes. It is worth noting other possible mechanisms: clustering may also activate Aurora B independent of phosphorylation, as is the case for kinases such as Raf and EGFR (Goetz et al., 2003; Zhang et al., 2006), or chromatin

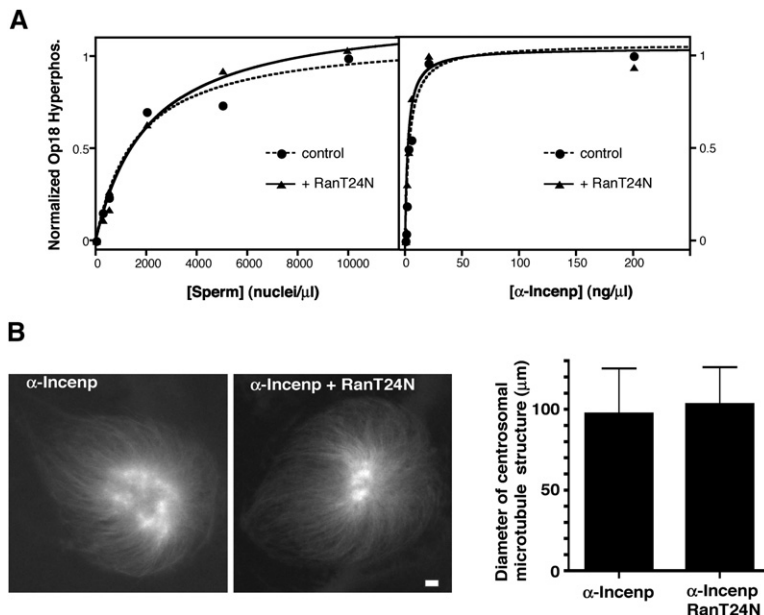


Figure 7. The Aurora B Pathway Is Activated and Can Promote Microtubule Assembly by a Ran-Independent Mechanism

(A) Nocodazole-treated CSF extract was incubated with the indicated concentrations of either sperm nuclei (left) or anti-Incenp antibody (right) at 20°C for 60 min, in the presence (triangle) or absence (circle) of 15 μM RanT24N (a gift of E. Arias). The degree of hyperphosphorylation of Op18 was determined by quantitative western blot. Data points were fit to a hyperbolic dose-response curve.

(B) Centrosomes (500/μl) were added to CSF extract that had been preincubated with 10 ng/μl anti-Incenp antibodies at 4°C for 70 min and incubated at 20°C for 70 min in the presence or absence of 15 μM RanT24N. Representative images of rhodamine-tubulin are shown. The scale bar is 10 μm. The average diameter with standard deviation of microtubule structures associated with monoasters in each sample is shown. n= 134 and 98, respectively.

or its associated molecules might directly induce a non-clustering-mediated structural change in Aurora B.

It is also possible that chromatin exerts its effect on the Aurora B pathway by inhibiting protein phosphatase activities. However, our data indicate that chromatin directly stimulates the kinase activity of Aurora B, since we demonstrate that Dasra proteins (which are required for loading of the CPC onto chromatin) are needed for spindle assembly. Importantly, more than 90% of Dasra A is associated with Incenp and Aurora B in the cytoplasm of *Xenopus* egg extracts (Sampath et al., 2004). In addition, it has been reported that recombinant human Dasra B/Borealin does not affect the in vitro kinase activity of Aurora B (Gassmann et al., 2004). Thus, it is unlikely that Dasra proteins stimulate the enzymatic activity of Aurora B simply by virtue of their interactions.

The spatial distribution of phosphorylated substrates around chromatin can be finely regulated by the level of phosphatase activity, and substrate diffusibility and stability, whereas the amplitude of the gradient is most sensitive to kinase activity (Kholodenko et al., 2000). For example, the freely diffusible Op18-tubulin interaction is abrogated in the vicinity of chromosomes (4–8 μm) by a gradient of Op18 phosphorylation, the extent of which is mainly determined by phosphatase activity/concentration and the Op18 diffusion rate (Niethammer et al., 2004). Alternatively, if the substrate is immobilized on chromosomes, kinase activity dictates the behavior of the phospho-substrate. MCAK, a protein that is bound to centromeric chromatin, is more efficiently phosphorylated at Ser196 by Aurora B on centromeres of unaligned chromosomes than on aligned chromosomes (Lan et al., 2004). This raises the question of whether a change in chromatin status between sister kinetochores can effectively regulate Aurora B activity by modulating its local concentration. In summary, our results illustrating that Aurora B is activated by increased

local concentration have important implications for the several roles of this complex throughout mitosis.

Importance of Chromatin-Dependent Activation of the Aurora B Pathway in Spindle Assembly

Integrating centrosomal and chromosomal microtubules into one spindle is a particularly important task for the egg, since the diameter of the cell at the first mitotic division in *Xenopus laevis* is ~1 mm, as compared to the ~30 μm spindle. Centrosomal microtubules are highly unstable in metaphase egg extracts, and they are not capable of forming bipolar spindles in the absence of associated chromosomes (Sawin and Mitchison, 1991). Using an antibody against Incenp, however, we were able to stimulate Aurora B-mediated phosphorylation in a chromatin-independent manner, resulting in the formation of chromosome-free bipolar spindle-like structures by centrosomes. From these observations, we speculate that local activation of Aurora B-dependent phosphorylation around chromosomes acts to couple spindle assembly initiated by chromatin and centrosomes. This function of Aurora B might be related to the “long-range communication” previously demonstrated to occur between centrosomes and chromatin (Carazo-Salas and Karsenti, 2003), as localized Aurora B activation by chromatin could promote the polarizing effect of chromatin on centrosome growth.

We have shown that the functions of the Dasra proteins in spindle microtubule assembly can be bypassed by the addition of anti-Incenp antibodies. Dasra-dependent recruitment of the CPC to chromatin appears to make the Aurora B pathway responsive to chromatin, but alternative mechanisms, such as the Ran-GTP pathway, can be engaged, permitting microtubule assembly on chromosomes if the Aurora B pathway is activated in a chromatin-independent manner. Indeed, we have previously shown that the Ran-GTP pathway remains active even in the

absence of the CPC (Sampath et al., 2004). Here, we showed that chromosomes were able to activate the Aurora B pathway in the presence of dominant-negative Ran. These results altogether indicate that the Ran-GTP pathway and the Aurora B pathway are both activated by chromatin, and that they cooperate to assemble bipolar spindles.

We currently do not understand the biological significance of centrosomal activation of the Aurora B pathway, since centrosomal microtubule size was not largely affected in Δ Incnp extracts (Figure 6). However, if the Aurora B pathway is artificially activated in the cytoplasm, spindle assembly is not restricted to chromosomes and can be elicited by centrosomes alone. Intriguingly, achromosomal spindles are also assembled by beads coated with anti-Aurora A antibody in the presence of Ran-GTP (Tsai and Zheng, 2005). These results suggest that the Aurora pathways do not merely promote microtubule assembly, but may contribute to establishing microtubule bipolarity.

In conclusion, we have demonstrated the importance of spatial regulation of the Aurora B pathway during spindle assembly. Previous studies have shown that chromatin creates a chromosome-centered Ran-GTP gradient responsible for the localized activation of microtubule-assembly factors around chromosomes (Caudron et al., 2005). While the catalytic activity of Rcc1 for Ran-GTP generation is directly induced by the H2A/H2B interaction (Nemergut et al., 2001), we suggest that local enrichment of the CPC on chromatin drives Aurora B pathway activation. In light of the data presented here, it is interesting to consider why two pathways must be regulated by chromatin in order to generate a spindle. Of note, the two pathways differ in the effects of their downstream targets; while two major microtubule-catastrophe regulators, MCAK and Op18, are inhibited by Aurora B, microtubule assembly factors such as TPX2, NuMA, and NuSAP are activated by Ran-GTP. Each downstream protein may have structural preferences or limitations for its regulation by phosphorylation or Ran-GTP/Importin β . In addition, having two independent mechanisms may contribute to the stability and dynamicity of the system. Understanding the integration between the Ran-GTP and Aurora B pathways will be crucial to elucidating the principles governing microtubule assembly around chromatin.

EXPERIMENTAL PROCEDURES

See the [Supplemental Data](#) for a detailed description of the experimental procedures.

Immunodepletion from RNase-Treated *Xenopus* Egg Extracts

Control rabbit IgG or anti-xIncnp (Sampath et al., 2004) were cross-linked to protein-A Dynabeads (Invitrogen) by DMP (dimethyl pimelimidate dihydrochloride, Pierce). To prepare RNase-treated *Xenopus laevis* egg extracts, a method described by Murray (1991) was applied. Extracts were depleted twice by using anti-xIncnp beads. In the second round of depletion, protein-A Dynabeads were added to remove any leached antibody that remained in the extract.

We found that chemical crosslinking of the antibodies to beads by DMP was critical for mRNA-dependent rescue experiments, as anti-

bodies that were passively conjugated to beads without crosslinking leached into extracts during immunodepletion and caused activation of the xAurora B pathway in an xIncnp-dependent manner. Although our previous report (Sampath et al., 2004) used a noncrosslinked method for immunodepletion, the conclusion was not affected by leached antibodies, as little xIncnp remained in depleted extracts, and no activation of the xAurora B pathway was seen (A.E.K. and S.C.S., unpublished data).

Spindle Assembly in RNase-Treated, Immunodepleted Egg Extracts

All incubations for spindle assembly with immunodepleted egg extracts were carried out at 20°C. For a typical spindle assembly on replicated chromosomes, 20 μ l RNase-treated control or immunodepleted egg extract containing sperm nuclei (final concentration 500/ μ l) or purified centrosomes (a gift of K. Kinoshita), 2 μ l mRNA, and 0.3 mM calcium chloride were incubated for 80 min at 20°C to prepare interphase extracts. Metaphase spindles were assembled by the addition of 40 μ l fresh RNase-treated control or immunodepleted extract, followed by incubation at 20°C for 60 min. To score phenotypes, 3 μ l Fix (Murray, 1991) was added to 1 μ l extract on a slide, and the process was repeated three times.

For spindle assembly and western blot analysis with chromatin beads, 5 μ l DNA-coated beads per sample, prepared as previously described (Heald et al., 1998), were washed once with 20 μ l RNase-treated control or immunodepleted extract, added to 20 μ l RNase-treated control or immunodepleted extract containing rhodamine-tubulin, and incubated for 80 min at 20°C after the addition of 0.3 mM calcium chloride. M phase entry was induced as described above.

Microscopy

Spindles assembled in *Xenopus* egg extract were processed for immunofluorescence as described (Desai et al., 1999a). Affinity-purified anti-xIncnp antibodies, anti-xDasra A antibodies, and control IgG were used at 2 μ g/ml in AbDil (TBS, 0.1% Triton X-100, 2% BSA). Alexa 488-conjugated goat anti-rabbit antibody (Molecular Probes) was used for detection. Hoechst 33258-stained chromosomes, rhodamine-tubulin-labeled microtubules, and fluorescent antibodies were imaged with a Carl Zeiss Axioplan 2 microscope equipped with a Photometrics CoolSnap HQ-cooled CCD camera and controlled by MetaMorph software (Universal Imaging). Images were processed with MetaMorph and Adobe Photoshop. To obtain the size of centrosomal microtubule structures, images were acquired with a Plan Neofluar 20 \times Objective, and the diameter of the major and minor axes of each microtubule-occupied region was measured with MetaMorph.

Conventional Western Blots

Primary and secondary antibodies were diluted in PBS/4% nonfat milk at the following concentrations: 5 μ g/ml anti-xDasra A (Sampath et al., 2004), 5 μ g/ml anti-xIncnp, 5 μ g/ml anti-xAurora B (raised against a C-terminal peptide, CRRVLPVYQSTQSK), 5 μ g/ml anti-Plx1 (raised against a C-terminal peptide, CQSSKSAVAHVKA), 4 μ g/ml anti-histone phospho-H3S10 (Abcam [ab14955] or Millipore [06-570]), 0.2 μ g/ml anti-Op18 (a gift of R. Heald), 1:5000 anti- α -tubulin (DM1, Sigma), 3 μ g/ml anti-xKid antibody (Funabiki and Murray, 2000). Antibodies were detected with either ECL (Amersham; for anti-xDasra A, anti-xIncnp, anti-Op18, anti-Plx1, anti-tubulin, anti-xKid) or Visualizer (Upstate; for anti-xAurora B).

Quantitative Western Blots

Procedures are similar to the conventional western blot, except that 0.1% Tween 20 was added to primary and secondary antibodies diluted in PBS/4% nonfat milk. IRDye 680 goat anti-rabbit IgG (Li-Cor) and IRDye 800CW goat anti-mouse IgG (Li-Cor) were used as secondary antibodies. The Odyssey Infrared Imaging System (Li-Cor) was used for detection and quantitation. Op18 hyperphosphorylation was measured by calculating the total fluorescence of

the slowest-migrating band for each sample by using xKid (Funabiki and Murray, 2000) or α -tubulin as a loading normalization.

Quantitative Mass Spectrometry

To purify the preactivated and postactivated forms of CPCs from metaphase egg extracts, CSF extracts were incubated with anti-xIncnp antibodies crosslinked to Dynabeads at 4°C and 20°C for 75 min. In addition, CSF extracts to which okadaic acid (0.5 μ M) or DMSO (0.5% v/v) was added, were incubated at 20°C for 30 min, cooled to 4°C for 10 min, mixed with anti-xIncnp beads, and incubated further for 70 min at 4°C. Immunoprecipitated complexes were separated by SDS-PAGE and were visualized by GelCode blue staining (Pierce). The band corresponding to xIncnp was excised, destained, alkylated, and digested with trypsin by following published methods (Chang et al., 2004). xIncnp tryptic peptides were then simultaneously extracted from the gel slice and were propionylated for the purpose of quantifying phosphorylation by mass spectrometry, as described previously (Jin et al., 2005). Briefly, the 4°C and DMSO samples were treated with a sequence of solvents containing 1% d₁₀-propionic anhydride, while the 20°C and okadaic acid samples were treated with solvents containing 1% d₀-propionic anhydride. The resulting differentially labeled peptides were dried and crystallized with 2,5-dihydroxybenzoic acid (DHB) MALDI matrix and were subjected to single-stage and tandem mass spectrometry for relative quantification of peptide phosphorylation (Chang et al., 2004).

Supplemental Data

Supplemental Data include nine figures and additional experimental procedures and are available at <http://www.developmentalcell.com/cgi/content/full/12/1/31/DC1/>.

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